Form PTO-1390 (REV 10-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
1619.0110000/SRL/KYP	
U.S. APPLICATION NO (IF KNOWN, SEE 37 C.F.R. § 1.5)	
To be assigned 10/070664	
INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/01052	September 8, 2000	September 9, 1999

TITLE OF INVENTION

Diagnosis, Prognosis and Treatment of Trinucleotide Repeat-Associated Diseases and Intranuclear Inclusions-Associated Diseases

APPLICANT(S) FOR DO/EO/US

Guy ROULEAU, Bernard BRAIS, Merdhad JANNATIPOUR, and Claudia GASPAR

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ■ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ■ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ■ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 372(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ■ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/070664		

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... **$ 100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 520.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	26- 20 =	6	X **$18.00**	$ 54.00	
Independent Claims	3- 3 =	0	X **$84.00**	$	
Multiple dependent claim(s) (if applicable)			+ **$280.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$	
■ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$	
SUBTOTAL =				$54.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property			+	$	
TOTAL FEES ENCLOSED =				$574.00	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $574.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

CUSTOMER NUMBER 26111
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
1100 New York Avenue, NW, Suite 600
Washington, D.C. 20005-3934

SIGNATURE

Steven R. Ludwig
NAME

36,203
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Rouleau *et al.*	Art Unit: *To be assigned*
Appl. No. *To be assigned* (*Nat'l Phase of Int'l Appl. No. PCT/CA00/01052*)	Examiner: *To be assigned*
Filed: March 8, 2002 *(Int'l Filing Date: September 8, 2000)*	Atty. Docket: 1619.0110000/SRL/KYP
For: **Diagnosis, Prognosis and Treatment of Trinucleotide Repeat-Associated Diseases and Intranuclear Inclusions-Associated Diseases**	

Preliminary Amendment

Commissioner for Patents
Washington, D.C. 20231

Sir:

Please enter the following amendments prior to examination of the above-referenced application. This Amendment is provided in the following format:

(A) A clean version of each replacement paragraph/section/claim along with clear instructions for entry;

(B) Starting on a separate page, appropriate remarks and arguments. 37 C.F.R. § 1.111 and MPEP 714; and

(C) Starting on a separate page, a marked-up version entitled: "Version with markings to show changes made."

It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent

abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Amendments

In the Claims:

Please cancel claims 3-4, 7-10, 13, and 15 without prejudice or disclaimer.

Please add the following claims:

16. (new) The method of claim 1, wherein said disease is a neurological disease.

17. (new) The method of claim 2, wherein said disease is a neurological disease.

18. (new) The method of claim 1, wherein said determining is carried out with one of a ligand and/or a nucleic acid sequence.

19. (new) The method of claim 2, wherein said determining is carried out with one of a ligand and/or a nucleic acid sequence.

20. (new) The method of claim 16, wherein said determining is carried out with one of a ligand and/or a nucleic acid sequence.

21. (new) The method of claim 17, wherein said determining is carried out with one of a ligand and/or a nucleic acid sequence.

22. (new) The method of claim 5, wherein said polyamino acid stretch-containing protein is expressed by an expression vector which comprises a repeat domain.

23. (new) The method of claim 6, wherein said polyamino acid stretch-containing protein is expressed by an expression vector which comprises a repeat domain.

24. (new) The method of claim 22, wherein said polyamino acid stretch-containing protein is a polyalanine stretch-containing protein.

25. (new) The method of claim 23, wherein said polyamino acid stretch-containing protein is a polyalanine stretch-containing protein.

26. (new) The method of claim 24, wherein said polyalanine stretch is encoded by a CAG repeat.

27. (new) The method of claim 25, wherein said polyalanine stretch is encoded by a CAG repeat.

28. (new) The method of claim 26, wherein said CAG repeat is an uninterrupted CAG tract.

29. (new) The method of claim 27, wherein said CAG repeat is an uninterrupted CAG tract.

30. (new) The method of claim 24, wherein said polyalanine stretch is encoded by a GCG repeat.

31. (new) The method of claim 25, wherein said polyalanine stretch is encoded by a GCG repeat.

32. (new) The method of claim 14, wherein said GCG repeat is present in the PABP2 gene.

33. (new) The method of claim 30, wherein said GCG repeat is present in the PABP2 gene.

34. (new) The method of claim 31, wherein said GCG repeat is present in the PABP2 gene.

Remarks

Upon entry of the foregoing amendment, claims 1-2, 5-6, 11-12, 14, 16-34 are pending in the application, with 1, 5, and 12 being the independent claims. Claims 3-4, 7-10, 13, and 15 are sought to be cancelled without prejudice to or disclaimer of the subject matter therein. New claims 16-34 are sought to be added. These changes are believed to introduce no new matter, and their entry is respectfully requested.

If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Steven R. Ludwig
Attorney for Applicants
Registration No. 36,203

Date: 3/8/02

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Version with markings to show changes made

Claims 3-4, 7-10, 13, and 15 are cancelled.

Claims 16-34 are added.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 9379
ROULEAU *et al.*	Art Unit: To be assigned
Appl. No. 10/070,664 (which is the U.S. Natl. Stage of PCT Appl. No. PCT/CA00/01052)	Examiner: To be assigned
I.A. Filing Date: September 8, 2000	Atty. Docket: 1619.0110000/SRL/AGU
For: **Diagnosis, Prognosis and Treatment of Trinucleotide Repeat-Associated Diseases and Intranuclear Inclusions-Associated Diseases**	

Second Preliminary Amendment and Submission of Sequence Listing

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notice to File Missing Parts of Application, dated June 10, 2002, in the above identified matter, and in advance of prosecution, please amend the application as follows:

In the Specification:

Please insert the sequence listing at the end of the application.

Remarks

No new matter has been added. The specification has been amended to direct the entry of this sequence listing after the claims of the above identified application.

In accordance with 37 C.F.R. § 1.821(g), this submission includes no new matter.

In accordance with 37 C.F.R. § 1.821(f), the paper copy of the Sequence Listing and the computer readable copy of the Sequence Listing submitted herewith in the above application are the same.

Summary

It is respectfully believed that this application is now in condition for examination. Early notice to this effect is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Angelique G. Uy
Agent for Applicants
Registration No. 48,832

Date: 10/10/02

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10/070664
JC19 Rec'd PCT/PTO 08 MAR 2002
PCT/CA00/01052

SEQUENCE LISTING

SEQ ID NO:1 TTTTAAGCTTAGACAAATAAACATGGAG

SEQ ID NO:2 CCGGTGGATCCCTCATCCTGATAGGTCCCGCTGCTG

SEQ ID NO:3 CCGGTGGATCCCTCATTGATAGGTCCCGCTGCTG

SEQ ID NO:4 TTTAAGCTTCCCACCATGGAGTCATCTTCCA

SEQ ID NO:5 CCGGTGGATCCCTCAGGGCGTAGTCGGGGACGTCG-TAGGGGTACATGGATGTGAACTCTGTCCTGATAGGTCCCG CTG

SEQ ID NO:6: CCGGTGGATCCCAGGGCGTAGTCGGGGACGTCGTAGGG-GTACATGGATGTGAACTCTGTCCTGATAGGTCCCGCTG

SEQ ID NO:7 CGGAAGAGACGAGAAGCCTACTCCGGAAAAACAGCAGCA-AAAGCAGC

TITLE OF THE INVENTION

DIAGNOSIS, PROGNOSIS AND TREATMENT OF TRINUCLEOTIDE REPEAT-ASSOCIATED DISEASES AND INTRANUCLEAR INCLUSIONS-ASSOCIATED DISEASES

FIELD OF THE INVENTION

The present invention relates to neurodegenerative disorders. More particularly, the invention relates to Machado-Joseph disease (MJD). The present invention also relates to trinucleotide repeat expansions and more particularly to CAG repeats, also termed expansions of a coding CAG repeat (exp-CAG), and GCG repeats. More particularly, the invention relates to: (1) exp-CAG associated diseases; (2) intranuclear inclusions (INI) in patients and cellular models of exp-CAG associated diseases; and (3) the elucidation of the mechanism responsible for the toxic effects of such repeats. The present invention therefore relates to the diagnosis, prognosis and treatment of repeat-associated diseases and INI-associated diseases as well as to assays for the identification of agents which could be used for the treatment of such diseases or disorders.

BACKGROUND OF THE INVENTION

Coding CAG triplet repeat expansions cause several neurodegenerative disorders, including Machado-Joseph disease (MJD)[1-5]. The presence of intranuclear filamentous inclusions (INI) containing expanded protein in MJD, as well as in other expanded CAG repeat disorders (exp-CAG), have lead to a nuclear toxicity model [1, 6-9]. Similar INI are found in oculopharyngeal muscular dystrophy, which is caused by a short expansion of an alanine encoding GCG repeat. According to the present invention, it is proposed that transcriptional or translational frameshifts occurring within expanded CAG tracts result in the production and accumulation of polyalanine-containing mutant proteins. These alanine polymers might deposit in cells forming INI and lead to

nuclear toxicity. Support for this disease model is provided using lymphoblast cells from MJD patients, as well as in pontine neurons of MJD brain and in *in vitro* cell culture models of the disease. Evidence that alanine polymers alone are toxic to cells is also provided and strongly suggests that a similar pathogenic mechanism underlies the other CAG repeat disorders.

Indeed, recent reviews describe a significant number of neurodegenerative diseases, including Huntington disease as well as spinal cerebellar ataxias which are caused by CAG repeat expansions. Of note, CAG repeats code for polyglutamine in the protein containing same. It is commonly believed that these polyglutamine stretches in proteins are toxic to cells, and these repeats are also termed CAG/polyglutamine repeats (Iver et al. 1999, Nature Medicine 5:383-384). These diseases, also termed polyglutamine diseases, are thought to occur by "a gain function mechanism". Unfortunately, the mechanism explaining toxicity of the polyglutamine diseases, apparently through an aggregation in nuclear inclusions, has yet to be provided, although transgenic mice bearing a polyglutamine repeat in a recombinant protein were shown to display intranuclear polyglutamine inclusions (Hardy et al. 1998, Science 282:1076-1079). Of relevance, although the pathogenic effect of these inclusion bodies is not clearly understood, it is recognized that numerous types of genes can contain these so-called CAG repeats, and that while these repeats are linked to the disease, the genes containing these repeats are "largely irrelevant to the disease process" (Hardy et al. ipid., *supra*).

INI are also found in oculopharyngeal muscular dystrophy (OPMD)[10], which is caused by short expansions of a polyalanine (polyAla) encoding GCG tract in the *PABP2* gene[11] (also see PCT/CA98/01133). In contrast to the CAG repeat disorders, where expansions frequently involve the addition of 20 or more codons, very small GCG expansions (exp-GCG) of 2 to 7 additional codons are seen in dominant OPMD, suggesting that polyalanine tracts are prone to aggregation and may be very toxic[11]. This contention is supported

by the observation that polyAla peptides containing more than 9 alanines (Ala) in a row form β-pleated sheet fibrillar macromolecules spontaneously *in vitro*[12], which in turn are extremely resistant to chemical and enzymatic degradation[13].

Short trinucleotide repeat expansions causing a human disease have been first described in PCT application number PCT/CA98/0113, of Rouleau et al., which teaches that the addition of only two GCG repeats (which encode the amino acid alanine [ALA]) is sufficient to cause dominant OPMD. OPMD expansions do not share the cardinal features of "dynamic mutations". The GCG expansions are not only short they are also meiotically quite stable. Furthermore, there is a clear cut-off between the normal and abnormal alleles: a single GCG expansion causing a recessive phenotype. The PAB II (GCG)7 allele was thus the first example of a relatively frequent allele which can act as either a modifier of a dominant phenotype or as a recessive mutation. A dosage effect of these repeats is also disclosed in PCT/CA98/01133, since a patient having an expansion in the polyalanine tract of the HOXD13 protein (Akarsu,. et al., 1996, Hum. Mol. Genet. 5: 945-952) has more severe deformities. A duplication causing a similar polyalanine expansion in the subunit 1 gene of the core-binding transcription factor CBF(1) has also been found to cause dominant cleido-cranial dysplasia (Mundlos, S. et al.,1997, Cell 89:773-779). Of note, however, the mutations in these two rare diseases are not triplet-repeats. They are duplications of "cryptic repeats" composed of mixed synonymous codons and are thought to result from unequal crossing over (Warren, 1997, Science 275: 408-409). In the case of OPMD, slippage during replication causing a reiteration of the GCG codon is a more likely mechanism (Wells, 1996, J. Biol. Chem. 271: 2875-2878).

Different observations converge to suggest that a gain of function of PAB II may cause the accumulation of nuclear filaments observed in OPMD (Tome et al., 1980, Acta Neuropath. 49: 85-87). PAB II is found mostly in dimeric and oligomeric form (Nemeth, et al.,1995, Nucleic Acids Res.23: 4034-4041). It is possible that the polyalanine tract plays a role in polymerization.

Polyalanine stretches have been found in many other nuclear proteins such as the HOX proteins, but their function is still unknown (Davies, et al., 1997, Cell90: 537-548). Alanine is a highly hydrophobic amino acid present in the cores of proteins. In dragline spider silk, polyalanine stretches are thought to form B-sheet structures important in ensuring the fibers' strength (Simmons, A.H. et al., Science 271:4-87 (1996)). Polyalanine oligomers have also been shown to be extremely resistant to chemical denaturation and enzymatic degradation (Forood et al. 1995, Bioch. and Biophy. Res. Com. 211:7-13). Their role in the disease process, however, has still not been clearly identified. The more severe phenotypes observed in homozygotes for the (GCG)9 mutations and compound heterozygotes for the (GCG)9 mutation and (GCG)7 allele may correspond to the fact that in these cases PAB II oligomers are composed only of mutated proteins. The ensuing faster filament accumulation could cause accelerated cell death. The recent description of nuclear filament inclusions in Huntington's disease, raises the possibility that "nuclear toxicity" caused by the accumulation of mutated homopolymeric domains is involved in the molecular pathophysiology of other triplet repeat diseases (Davies, S.W. et al., Cell 90:537-548 (1997); Scherzinger, E. et al., Cell 90:549-558 (1997); DiFiglia, M. et al., Science 277:1990-1993 (1997)). Additional data, including immunocytochemical and expression studies will have to be provided to test this pathophysiological hypothesis and provide some insight into why certain muscle groups are more affected, while all tissues express PAB II.

There thus remains a need to elucidate the mechanism by which GCG and CAG expansions are toxic to cells. There also remains a need to provide diagnosis and/or prognosis and/or treatment tools for diseases associated with GCG or CAG repeats.

The present invention seeks to meet these and other needs.

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The invention therefore concerns the identification of the mechanistic action of expanded CAG tracts in cell pathogenesis, cell death or disease. More specifically, the invention relates to the identification that mutational events within these CAG enable them to encodepolyalanine stretches which accumulate in intranuclear filamentous inclusions and somehow trigger toxicity in cells. In particular, the present invention relates totranslational and/or transcriptional frameshift events occurring within the CAG tracts, thereby resulting in the production and accumulation of polyalanine-containing mutant proteins.

The present invention, in addition, relates to a formal identification of the toxic effects ofpolyalanine stretches on nuclear toxicity. The Applicant is thus the first to have demonstrated that CAG expansions can give rise to production of mutant proteins containing polyalanine stretches. The Applicant is also the first to have demonstrated that polyalanine stretches in proteins are indeed toxic to cells.

The instant invention also relates to gene replacement technologies aimed at deleting a repeat-containing protein giving rise to a mutant protein by a normal corresponding protein (i.e. lacking the repeat or having a smaller repeat).

The present invention also provides the means to determine a predisposition to developing a disease or condition associated with the expression of a polyamino acid-containing protein such as polyalanine-containing proteins. This determination could thus enable a better prognosis of the disease and condition and enable a determination of the best treatment or prevention of the disease or condition.

Another aim of the present invention is thus to provide means to screen humans (and more broadly animals) to identify those that might have a predisposition to developing a disease associated with the expression of genes

that lead to polyalanine-containing proteins such as CAG, GCG-repeat containing genes.

It is thus an aim of the present invention to provide the means to better manage such disease prevention and intervention programs.

Before the present invention, CAG repeat expansions (also known as CAG repeats or CAG/polyglutamine stretches) were recognized as a common denominator in numerous neurological diseases and were thought to code for polyglutamine stretches in the mutant protein. These polyglutamine stretches were thought to confer "a gain of toxic property to these proteins" (Iver et al. *supra*) by a mechanism that was not understood (Hardy et al. *supra*). Indeed, CAG tract toxicity was also referred to as polyglutamine diseases. The present invention demonstrates that these CAG repeats actually encode polyalanine stretches.

Prior to the present invention, the identification of CAG repeats in a gene correlating with a neurological disease led to the classification of such disease in the polyglutamine diseases (Hardy et al. *supra*). The present invention now demonstrates that polyalanine stretches, as opposed to polyglutamine stretches, are responsible for the diseases.

In view of the above, the present invention opens the way to numerous methods of diagnosing, prognosing or treating CAG repeat-dependent diseases or conditions. In addition, it provides means to diagnose, prognose and treat diseases or conditions associated with polyalanine-containing proteins (i.e. GCG repeats). Non-limiting examples thereof comprise methods usingligands (i.e. polyclonal and monoclonal antibodies), nucleic acid sequences, restriction length polymorphisms (RFLPs) and the like.

While the instant invention is more particularly directed to neurological diseases, as is demonstrated with Machado-Joseph disease, it should be understood that the present invention should not be so limited. Indeed,

polyalanine stretches have been shown to be responsible for non-neurological diseases such as, for example, a muscle disease (OPMD; PCT/CA98/01133).

In order to better understand the disease process associated with the presence of INI in exp-CAG and exp-GCG associated disorders, a direct assessment of whether polyAla stretches accumulated as intranuclear protein aggregates was carried out. Experiments were thus performed to analyze whether rare transcriptional or translational frameshifts in large CAG stretches resulting in new reading frames with the formation of a hybrid protein containing a mixed polyglutamine/poly-alanine tract occurred. Additionally, it was of importance to assess whether the resultant polyAla peptides accumulate in nuclei where they form INI.

Surprisingly, it was discovered that an antiserum raised against the hypothetical COOH terminus of the predicted polyAla containing frameshifted ataxin-3 protein (MJD-Ala) detects the frameshifted species in lymphoblastoid cells from MJD patients with large CAG tracts. This antiserum detects these polyAla tracts as insoluble macromolecules on Western blots, and as intranuclear inclusions by immunocytochemistry. Frameshifted species were also present in INI in pontine neurons of MJD brain. Transfection of COS-7 cells with full-length *MJD-1* fused to the enhanced green fluorescence protein (*EGFP*) gene in the alternative polyAla reading frame also leads to EGFP accumulation preferentially when the CAG tract is expanded.

Of interest, it is also demonstrated that long CAG repeats are prone to frameshifts, which result in accumulation of the predicted polyAla-containing inclusions. Transfection of COS-7 cells with mutated *MJD-1* constructs containing alanine-coding GCA stretches results in a more severe phenotype when compared to their CAG counterparts. Furthermore, transfected polyAla-encoding GCA stretches alone are toxic and form aggregates.

How these accumulations lead to cell death still needs to be elucidated.

A frameshift error occurring within a CAG tract thus results in the alternate alanine-encoding GCA frame. Many authors have reported frameshifts at the level of transcription or translation. The observation of transcriptional errors of the β-amyloid precursor protein and ubiquitin-B in Alzheimer's disease[14], and apoB86[15], supports the existence of such errors, and their role in disease pathogenesis. Translational errors have also been shown to occur and may be the basis for the formation of frameshifted proteins[16].

In accordance with one embodiment of the present invention, there is therefore provided a method for the diagnosis of a disease associated with protein accumulation in intranuclear inclusions, which comprises obtaining a sample of a patient and determining a presence of the protein accumulation in the intranuclear inclusions, wherein this protein accumulation is indicative of a disease related thereto.

In accordance with another embodiment of the present invention, there is also provided a method for the screening of agents which can modulate at least one of (1) a polyamino acid-containing protein expression; (2) accumulation of polyamino acid-containing proteins in intranuclear inclusions; and (3) toxicity to cells, which comprises: a) incubating a cell harboring an expression vector of the present invention, comprising a repeat domain which can give rise to a polyamino acid-containing protein associated with a disease or condition in an animal, with a compound; and b) assessing one of (1) an expression of the polyamino acid-containing protein; (2) accumulation of the polyamino acid-containing protein; and (3) toxicity to cells; whereby a modulator is selected when the agent significantly modulates one of the expression, accumulation and toxicity, as compared to a control agent.

The instant invention also relates to GCG repeats encoding polyalanine stretches and their association with protein accumulation in a cell nucleus, swallowing difficulty and/or ptosis in a patient. In accordance with another embodiment of the present invention, there is provided a method for the

diagnosis or prognosis of a disease associated with protein accumulation in a cell nucleus, and/or swallowing difficulty and/or ptosis in a human patient, which comprises:

a) obtaining a sample of a patient; and

b) determining the extract of the polyalanine stretch in an alanine stretch-containing protein having the amino acid sequence:

Met $(Ala)_{6+n}$ Ala,

wherein n is selected from 0 to 7, and

whereby an n equal to 1 to 7 is indicative of a disease related with the protein accumulation in the nucleus, and/or a swallowing difficulty and/or ptosis in the patient. In a related aspect of the present invention, there is provided a human PAB II protein comprising a polymorphic GCG repeat encoding a polyalanine stretch having the sequence

Met $(Ala)_{6+n}$ Ala,

wherein n is 0, and wherein the sequence is indicative of a non-disease phenotype associated with protein accumulation in a cell nucleus, swallowing difficulty, and/or ptosis in a human patient.

In order to provide a clear and consistent understanding of terms used in the present description, a number of definitions are provided hereinbelow.

Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission.

Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell cultures, infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can

be found in reference manuals such as for example Sambrook et al. (1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratories) and Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, New York).

The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

As used herein, "nucleic acid molecule", refers to a polymer of nucleotides. Non-limiting examples thereof include DNA (i.e. genomic DNA, cDNA) and RNA molecules (i.e. mRNA). The nucleic acid molecule can be obtained by cloning techniques or synthesized. DNA can be double-stranded or single-stranded (coding strand or non-coding strand [antisense]).

The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering.

The term "DNA segment", is used herein, to refer to a DNA molecule comprising a linear stretch or sequence of nucleotides. This sequence when read in accordance with the genetic code, can encode a linear stretch or sequence of amino acids which can be referred to as a polypeptide, protein, protein fragment and the like.

The terminology "amplification pair" refers herein to a pair of oligonucleotides (oligos) of the present invention, which are selected to be used together in amplifying a selected nucleic acid sequence by one of a number of types of amplification processes, preferably a polymerase chain reaction. Other types of amplification processes include ligase chain reaction, strand displacement amplification, or nucleic acid sequence-based amplification, as explained in greater detail below. As commonly known in the art, theoligos are designed to bind to a complementary sequence under selected conditions.

The nucleic acid (i.e. DNA or RNA) for practicing the present invention may be obtained according to well known methods.

As used herein, the term "physiologically relevant" is meant to describe a frameshifting event which can result in the production of a toxic protein *in vivo*.

Oligonucleotide probes or primers of the present invention may be of any suitable length, depending on the particular assay format and the particular needs and targeted sequences employed. In general, the oligonucleotide probes or primers are at least 12 nucleotides in length, preferably between 15 and 24 molecules, and they may be adapted to be especially suited to a chosen nucleic acid amplification system. As commonly known in the art, the oligonucleotide probes and primers can be designed by taking into consideration the melting point of hydrizidation thereof with its targeted sequence (see below and in Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, CSH Laboratories; Ausubel et al., 1989, in Current Protocols in Molecular Biology, John Wiley & Sons Inc., N.Y.).

The terms "oligonucleotide" or "DNA" molecule or sequence refers to a molecule comprised of the deoxyribonucleotides adenine (A), guanine (G), thymine (T) and/or cytosine (C), in a double-stranded or single-stranded form. The term "oligonucleotide" or "DNA" can be found in linear DNA molecules or fragments, viruses, plasmids, vectors, chromosomes or synthetically derived DNA. As used herein, particular DNA sequences may be described according to the normal convention of giving only the sequence in the 5' to 3' direction.

"Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having complementary base sequences, which under appropriate conditions will form a thermodynamically favored double-stranded structure. Examples of hybridization conditions can be found in the two laboratory manuals referred above (Sambrook et al., 1989, *supra* and Ausubel et al., 1989, *supra*) and are commonly known in the art. In the case of a hybridization to a nitrocellulose filter, as for example in the well known Southern blotting procedure, a nitrocellulose filter can be incubated

overnight at 65°C with a labeled probe in a solution containing 50% formamide, high salt (5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 µg/ml denatured carrier DNA (i.e. salmon sperm DNA). The non-specifically binding probe can then be washed off the filter by several washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in view of the desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (Tm) of the DNA hybrid (Sambrook et al. 1989, *supra*). Of course, RNA-DNA hybrids can also be formed and detected. In such cases, the conditions of hybridization and washing can be adapted according to well known methods by the person of ordinary skill. Stringent conditions will be preferably used (Sambrook et al., 1989, *supra*).

Probes or primers of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and α-nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic acid molecule. Acids Res., 14:5019. Probes or primers of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA.

The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less preferred, labeled proteins could also be used to detect a particular nucleic acid sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like.

Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the sensitivity of the detection. Furthermore, it enables automation (the same can also be said of detection of

proteins using ligands such as antibodies). Probes can be labeled according to numerous well known methods (Sambrook et al., 1989, supra). Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma ^{32}P ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* in the presence of radioactive dNTP (i.e. uniformly labeled DNA probe using random oligonucleotide primers in low-melt gels), using the SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

As used herein, "oligonucleotides" or "oligos" define a molecule having two or more nucleotides (ribo or deoxyribonucleotides). The size of the oligo will be dictated by the particular situation and ultimately on the particular use thereof and adapted accordingly by the person of ordinary skill. An oligonucleotide can be synthetised chemically or derived by cloning according to well known methods.

As used herein, a "primer" defines an oligonucleotide which is capable of annealing to a target sequence, thereby creating a double stranded region which can serve as an initiation point for DNA synthesis under suitable conditions.

Amplification of a selected, or target, nucleic acid sequence may be carried out by a number of suitable methods. See generally Kwoh et al., 1990, Am. Biotechnol. Lab. 8:14-25. Numerous amplification techniques have been described and can be readily adapted to suit particular needs of a person

of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-based amplification, the Qβ replicase system and NASBA (Kwoh et al., 1989, Proc. Natl. Acad. Sci. USA 86, 1173-1177; Lizardi et al., 1988, BioTechnology 6:1197-1202; Malek et al., 1994, Methods Mol. Biol., 28:253-260; and Sambrook et al., 1989, *supra*). Preferably, amplification will be carried out using PCR.

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of eachprimer which is synthesized is complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analysed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization following EtBr staining of the DNA following gel electrophores, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990).

Ligase chain reaction (LCR) is carried out in accordance with known techniques (Weiss, 1991, Science 254:1292). Adaptation of the protocol to meet the desired needs can be carried out by a person of ordinary skill. Strand displacement amplification (SDA) is also carried out in accordance with known

techniques or adaptations thereof to meet the particular needs (Walker et al., 1992, Proc. Natl. Acad. Sci. USA 89:392-396; and ibid., 1992, Nucleic Acids Res. 20:1691-1696).

As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise the a specific polypeptide or protein. It will be readily recognized by the person of ordinary skill, that the nucleic acid sequence of the present invention can be incorporated into anyone of numerous established kit formats which are well known in the art.

A "heterologous" (i.e. a heterologous gene) region of a DNA molecule is a subsegment segment of DNA within a larger segment that is not found in association therewith in nature. The term 'heterologous" can be similarly used to define two polypeptidic segments not joined together in nature. Non-limiting examples of heterologous genes include reporter genes such as green fluorescence protein, luciferase, chloramphenicol acetyl transferase, β-galactosidase, and the like which can be juxtaposed or joined to heterologous control regions or to heterologous polypeptides.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

The term "expression" defines the process by which a gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or more.

The terminology "expression vector" defines a vector or vehicle as described above but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted

sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often refered to as being operably linked to control elements or sequences.

Operably linked sequences may also include two segments that are transcribed onto the same RNA transcript. Thus, two sequences, such as a promoter and a "reporter sequence" are operably linked if transcription commencing in the promoter will produce an RNA transcript of the reporter sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another.

Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

Prokaryotic expressions are useful for the preparation of large quantities of the protein encoded by the DNA sequence of interest. This protein can be purified according to standard protocols that take advantage of the intrinsic properties thereof, such as size and charge (i.e. SDS gel electrophoresis, gel filtration, centrifugation, ion exchange chromatography...). In addition, the protein of interest can be purified via affinity chromatography using polyclonal or monoclonal antibodies. The purified protein can be used for diagnostic or therapeutic applications.

The DNA construct can be a vector comprising a promoter that is operably linked to an oligonucleotide sequence of the present invention, which is in turn, operably linked to a heterologous gene, such as the gene for the luciferase reporter molecule. "Promoter" refers to a DNA regulatory region capable of binding directly or indirectly to RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of

the present invention, the promoter is bound at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter will be found a transcription initiation site (conveniently defined by mapping with S1 nuclease), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boses and "CCAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of a sequence whether an nucleic acid or amino acid sequence, a molecule that retains a biological activity (either function or structural) that is substantially similar to that of the original sequence. This functional derivative or equivalent may be a natural derivative or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid as chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophylicity and the like. The term "functional derivatives" is intended to include "fragments", "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the present invention.

Thus, the term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention.

The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art.

As used herein, "chemical derivatives" is meant to cover additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic of the derivative (i.e. solubility, absorption, half life and the like, decrease of toxicity). Such moieties are examplified in Remington's Pharmaceutical Sciences (1980). Methods of coupling these chemical-physical moieties to a polypeptide are well known in the art.

The term "allele" defines an alternative form of a gene which occupies a given locus on a chromosome.

As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. As well known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotide. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. A mutant polypeptide can be encoded from a mutant nucleic acid molecule. In addition, mutant proteins can be produced through aberrant events during replication, transcription and/or translation. Frameshifting (the switching from a particular reading frame to another) is such a mechanism that can modify the sequence of the translated protein.

As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a "purified protein" has been purified to a level not found in nature. A "substantially pure" molecule is a molecule that is lacking in all other cellular components.

As used herein, the terms "molecule", "compound", "agent", or "ligand" are used interchangeably and broadly to refer to natural, synthetic or

semi-synthetic molecules or compounds. The term "molecule" therefore denotes for example chemicals, macromolecules, cell or tissue extracts (from plants or animals) and the like. Non limiting examples of molecules include nucleic acid molecules, peptides, antibodies, carbohydrates and pharmaceutical/therapeutical agents. The agents can be selected and screened by a variety of means including random screening, rational selection and by rational design using for example protein or ligand modelling methods such as computer modelling. The terms "rationally selected" or "rationally designed" are meant to define, for example, compounds which have been chosen based on the configuration of the polyalanine domains of the present invention. As will be understood by the person of ordinary skill, macromolecules having non-naturally occurring modifications are also within the scope of the term "molecule". For example, peptidomimetics, well known in the pharmaceutical industry and generally referred to as peptide analogs can be generated by modelling as mentioned above. Similarly, in one embodiment, the polypeptides of the present invention can be modified to enhance or decrease their stability. It should be understood that in most cases this modification should not alter the biological activity of the polyalanine domain (its toxic effect or INI localization property). The molecules identified in accordance with the teachings of the present invention have a therapeutic value in diseases or conditions in which the physiology or homeastasis of the cell and/or tissue is compromised by a production of polyalanine-containing proteins or polypeptides. Alternatively, the molecules identified in accordance with the teachings of the present invention find utility in the development of more efficient molecule to lower and/or abrogate the toxicity of such proteins and/or to reduce or eliminate the production of such mutant proteins. It will be understood that agents can be screened, in accordance with the present invention, with libraries of compounds, using for example automated screening methods (e.g. array technologies).

The level of gene expression of the reporter gene (e.g. the level of luciferase, or β-gal, produced) within the treated cells can be compared to that of the reporter gene in the absence of the molecules(s). The difference between the levels of gene expression indicates whether the molecule(s) of interest agonizes the aforementioned interaction. The magnitude of the level of reporter gene product expressed (treated vs. untreated cells) provides a relative indication of the strength of that molecule(s) as an agonist. The same type of approach can also be used in the presence of an antagonist(s). Thus, modulators of the production of such proteins can be identified and selected. Non-limiting examples of modulators in accordance with the present invention include frameshift mutants or suppressors, relievers of codon rareness (i.e. relieving the limitation of rare codons which favor frameshifting events), agents which degrade polyalanine stretches, tRNAs, tRNA suppressors and the like. One skilled in the art will realize that the assays to identify compounds that modulate frameshifting of CAG repeats and the like, could be carried out using other repeats as well as other genes known to promote frameshifting. Such genes are known in the art.

The present invention also provides antisense nucleic acid molecules which can be used for example to modulate the expression of the mutant proteins of the present invention. An antisense nucleic acid molecule according to the present invention refers to a molecule capable of forming a stable duplex or triplex with a portion of its targeted nucleic acid sequence (DNA or RNA). The use of antisense nucleic acid molecules and the design and modification of such molecules is well known in the art as described for example in WO 96/32966, WO 96/11266, WO 94/15646, WO 93/08845 and USP 5,593,974. Antisense nucleic acid molecules according to the present invention can be derived from the nucleic acid sequences and modified in accordance to well known methods. For example, some antisense molecules can be designed to be more resistant to degradation to increase their affinity to their

targeted sequence, to affect their transport to chosen cell types or cell compartments, and/or to enhance their lipid solubility bu using nucleotide analogs and/or substituting chosen chemical fragments thereof, as commonly known in the art.

Alternatively, an indicator cell in accordance with the present invention can be used to identify antagonists. For example, the test molecule or molecules are incubated with the host cell in conjunction with one or more agonists held at a fixed concentration. An indication and relative strength of the antagonistic properties of the molecule(s) can be provided by comparing the level of gene expression in the indicator cell in the presence of the agonist, in the absence of test molecules vs in the presence thereof. Of course, the antagonistic effect of a molecule can also be determined in the absence ofagonist, simply by comparing the level of expression of the reporter gene product in the presence and absence of the test molecule(s).

It shall be understood that the *"in vivo"* experimental model can also be used to carry out an *"in vitro"* assay. For example, cellular extracts from the indicator cells can be prepared and used in one of the aforementioned *"in vitro"* tests.

As used herein the recitation "indicator cells" refers to, for example, cells that express a fusion protein comprising a polyalanine segment (e.g. a "CAG" repeat) and an identifiable or selectable phenotype or characteristic which enables an assessment of the level of fusion protein expression (e.g. a reporter protein). Such indicator cells can be used in the screening assays of the present invention. In certain embodiments, the indicator cells have been engineered so as to express a chosen derivative, fragment, homolog, or mutant of a repeat. It should be understood that the repeats should not be limited to CAG repeats. Indeed, GCG repeats can also be used. In addition, the invention should not be limited to polyalanine repeats, since the present invention provides for the testing of polyserine fragments and other polyamino acids which could be

expressed from a frameshifting event. The cells can be yeast cells or higher eukaryotic cells such as mammalian cells (WO 96/41169). Preferably, the indicator cells are higher eukaryotic cells. Non-limiting examples of such cells and vectors are exemplified herein below (i.e. Examples 2-4). In one particular embodiment, the indicator cell could be used to test a compound or a library thereof.

As exemplified herein below in one embodiment, a polyalanine polypeptide segment of the present invention is provided as a fusion protein. The design of constructs therefor and the expression and production of fusion proteins are exemplified herein and are well known in the art (Sambrook et al., 1989, *supra*; and Ausubel et al., 1994, *supra*).

Non limiting examples of such fusion proteins include a hemaglutinin fusions (HA) and Gluthione-S-transferase (GST) fusions. In certain embodiments, it might be beneficial to introduce a protease cleavage site between the two polypeptide sequences which have been fused. Such protease cleavage sites between two heterologously fused polypeptides are well known in the art.

In certain embodiments, it might also be beneficial to introduce a linker (commonly known) between the repeat segment of the protein and the heterologous polypeptide portion (e.g. reporter protein portion). Such fusion protein find utility in the assays of the present invention as well as for purification purposes, detection purposes and the like.

For certainty, the sequences and polypeptides useful to practice the invention include without being limited thereto mutants, homologs, subtypes, alleles and the like. It shall be understood that generally, the sequences of the present invention should encode a functional (albeit defective) repeat domain. It will be clear to the person of ordinary skill that whether a repeat domain of the present invention, variant, derivative, or fragment thereof retains its function in enabling a concentration of the protein containing same in INI or

triggering toxicity in cells or animals, can be readily determined by using the teachings and assays of the present invention and the general teachings of the art.

As exemplified herein below, the repeat domains of the present invention can be modified, for example by *in vitro* mutagenesis, to dissect the structure-function relationship thereof and permit a better design and identification of modulating compounds. However, some derivative or analogs having lost their biological function may still find utility, for example for raising antibodies. These antibodies could be used for detection or purification purposes. In addition, these antibodies could also act as competitive or non-competitive inhibitor and be found to be modulators of the biological activity of the repeat domain.

A host cell or indicator cell has been "transfected" by exogenous or heterologous DNA (e.g. a DNA construct) when such DNA has been introduced inside the cell. The transfecting DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, thetransfecting DNA may be maintained on a episomal element such as a plasmid. With respect to eukaryotic cells, a stably transfected cell is one in which the transfecting DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transfecting DNA. Transfection methods are well known in the art (Sambrook et al., 1989, *supra*; Ausubel et al., 1994 *supra*).

In general, techniques for preparing antibodies (including monoclonal antibodies and hybridomas) and for detecting antigens using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology", Elsevier Science Publisher, Amsterdam, The Netherlands) and in Harlow et al.,

1988 (in: Antibody- A Laboratory Manual, CSH Laboratories). The present invention also provides polyclonal, monoclonal antibodies, or humanized versions thereof, chimeric antibodies and the like which are specific to the repeat domains of the present invention.

From the specification and appended claims, the term therapeutic agent should be taken in a broad sense so as to also include a combination of at least two such therapeutic agents. Further, the therapeutic agent according to the present invention can be introduced into individuals in a number of ways. The therapeutic agent can also be delivered through a vehicle such as a liposome, which can be designed to be targeted to a specific cell type, and engineered to be administered through different routes. Having shown that a polyalanine segment is toxic to cells, the present invention provides the means to trigger toxicity in cells by expressing thereinto or delivering thereto a polyalanine-containing protein (or polyalanine-encoding nucleic acid). In accordance with known methods, a chosen cell population could be targeted.

For administration to humans, the prescribing medical professional will ultimately determine the appropriate form and dosage for a given patient, and this can be expected to vary according to the chosen therapeutic regimen (i.e. DNA construct, protein, cells), the response and condition of the patient as well as the severity of the disease.

Composition within the scope of the present invention should contain the active agent (i.e. fusion protein, nucleic acid, and molecule) in an amount effective to achieve the desired therapeutic effect while avoiding adverse side effects. Typically, the nucleic acids, fusion proteins and molecules in accordance with the present invention can be administered to mammals (i.e. humans) in doses ranging from 0.005 to 1 mg per kg of body weight per day of the mammal which is treated. Pharmaceutically acceptable preparations and salts of the active agent are within the scope of the present invention and are well known in the art (Remington's Pharmaceutical Science, 16th Ed., Mack Ed.). For

the administration of polypeptides, antagonists, agonists and the like, the amount administered should be chosen so as to avoid adverse side effects. The dosage will be adapted by the clinician in accordance with conventional factors such as the extent of the disease and different parameters from the patient. Typically, 0.001 to 50 mg/kg/day will be administered to the mammal.

The present invention also relates to a kit for diagnosing a disease or condition associated with the expression of a repeat domain, encoding for example a polyalanine stretch, or a predisposition to contracting same comprising a nucleic acid, a protein or a ligand in accordance with the present invention. For example, a compartmentalized kit in accordance with the present invention includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allow the efficient transfer of reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample (DNA protein or cells), a container which contains the primers used in the assay, containers which contain enzymes, containers which contain wash reagents, and containers which contain the reagents used to detect the extension products.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1 shows the Western blot analysis of lymphoblastoid cells from controls and MJD patients. a, Schematic representation of the MJD-Ala protein that results from a frameshift in the CAG tract showing the new C-terminus (italicized; used to raise the FS1 and FS2 antibodies). Western blots of two control lymphoblastoid cell lines (cLCL) and four MJD lymphoblastoid cell

lines (MJDLCL) immunoprobed with FS1 (b), anti-ataxin-3 (c), 1C2 (d) and FS1 pre-immune serum (e). Arrow indicates the threshold between stacking and resolving portions of the gel. Panels b-d represent serial probing of a single membrane.

Figure 2 shows the immunocytochemical detection of intranuclear deposits in lymphoblastoid cells. Immunocytochemistry of control LCL versus MJD LCL: absence of INI in control LCL probed with FS1 (a), and anti-ubiquitin (c), and detection of INI in MJD LCL with FS1 (b) and anti-ubiquitin (d). e, Immuno detection of MJD LCL with FS1 pre-immune serum. For all panels, the magnification before publication is 400x (left) and 1000x (right). These results have been replicated in three separate experiments.

Figure 3 shows the immunohistochemical detection of INI in MJD pontine neurons. Immunoprobing with FS1 antiserum in MJD pons (a) and control pons (b); immunoprobing with anti-ubiquitin in MJD pons (c) and control pons (d). INI in pontine neurons are indicated by arrowheads. Double labeling immunofluorescence analysis of MJD pons showing ubiquitin-labeled INI (e, h) and FS1-labeled INI (f, i), and the composite image of both labelings (g, j). For all panels, the magnification before publication is 1000x, before reproduction.

Figure 4 shows the constructs used in the transfection experiments. All constructs, with the exception of pMJD11, represent full-length *MJD-1*. Solid black boxes indicate the repeat portion of the constructs. Staggered ends indicate that EGFP will only be expressed if a frameshift occurs. Encircled blown up detail of pMJD1 is also present in pMJD2 and pMJD3; details of pMJD5 are present in pMJD6; details of pMJD7 are present in pMJD8 and details of pMJD9 are the same as pMJD10.

Figure 5 shows the transfection experiments with different MJD/EGFP constructs. a, DNA sequence of the clones with EGFP out of frame (pMJD1, pMJD2 and pMJD3) and b, with EGFP in glutamine frame (pMJD4), both (a, b) showing the predicted amino acid sequence. c-e, Fluorescence at 72 hours

of COS-7 cells transfected with pMJD1 (c), pMJD2 (d) and pMJD3 (e) where the MJD(CAG)$_n$/EGFP fusion protein is translated only when frameshifts to GCA-polyAla occur. f, COS-7 cells transfected with pMJD4 where EGFP is in frame with CAG-Gln. g, COS-7 cells transfected with the vector pEGFP-N1. h, perinuclear fluorescent aggregates observed in cells transfected with pMJD3. Pictures of sections c, d, e, f and g were taken at 25 x magnification for a fixed exposure time of 60 seconds before reproduction; picture shown in h, is at 400 x before reproduction. i-k, Western blots of protein isolated from cells shown in c, d, e, f, g and from mock-transfected cells immunoprobed with 1C2 (i), anti-ataxin-3 (j) and anti-HA (k). Arrows on the right of panel j indicate the proteins detected. Arrowhead on panel k indicates threshold between stacking and resolving portions of the gel. Rpts stands for repeats.

Figure 6 shows the time-course immunocytochemical analysis of COS-7 cells transfected with constructs encoding ataxin-3 with either a polyAla or a polyGln tract. Immunoprobing with anti-HA antibody at time-points 8 hours: (a) pMJD7, (b) pMJD9, (c) pMJD8, (d) pMJD10 and (e) vector pEGFP-N1 alone; 20 hours: (f) pMJD7, (g) pMJD9, (h) pMJD8, (i) pMJD10 and (j) vector pEGFP-N1 alone; 24 hours: (k) pMJD7, (l) pMJD9, (m) pMJD8, (n) pMJD10 and (o) vector pEGFP-N1 alone; 48 hours: (p) pMJD7, (q) pMJD9, (r) pMJD8, (s) pMJD10 and (t) vector pEGFP-N1 alone. For all panels pictures were taken at 1000x magnification, before reproduction.

Figure 7 shows the western analysis of transfected COS-7 cells (in figure 5). Blots were probed with (a) anti-HA, (b) 1C2. Arrow indicates threshold between stacking and resolving portions of the gel. Cells were harvested at 72 hours after transfection.

Figure 8 shows the immunocytochemical analysis of COS-7 cells transfected with a truncated polyAla-encoding construct. Immunoprobing with anti-HA antibody in: (a) cells transfected with 42A and (b) mocktransfected

cells. For all panels pictures were taken at 1000x magnification, before reproduction.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The data herein presented strongly suggest the fact that (1) frameshifts occur within CAG repeats and are responsible for the production of alanine-containing proteins, and (2) these proteins accumulate as toxic aggregates. Detection of the hypothetical peptide in lymphoblastoid and neuronal cells of MJD patients and not in controls, and the production of green fluorescence preferentially in cells transfected with *MJD-1* bearing large CAG tracts with an out of frame EGFP, supports the occurrence of rare frameshifts during transcription and/or translation of long CAG tracts resulting from the use of the alternative GCA/Ala reading frame. The relatively small proportion of frameshifted product may explain the absence of loss-of-function of the protein and the relatively late-onset of these diseases. This model of slow accumulation, due to relatively rare frameshift events, better explains the late-onset nature of these diseases given that, in light of the high expression of these proteins in affected brain regions, a glutamine toxicity model would be expected to result in much earlier cell death and disease onset[21-28].

Slippage into the third possible frame, AGC/Ser, may also be occurring. However, the absence of diseases associated with tracts of polyserine and the physical nature of alanine polymers, resulted in an exclusive focus on the GCA/Ala frame. While the relative frequencies of different frameshifts remain unknown, the fact that most translational frameshift errors cause a +2 shift in the frame[29] suggests that GCA/Ala may be the more frequent of the mutant species.

The present invention also assessed whether polyAla tracts are toxic and may be the initiating event in the formation of the INI seen in expanded CAG tract diseases. The evidence presented herein shows that in cells transfected with the CAG/Gln constructs, frameshifting into the alanine frame is progressive and frameshifted products are slowly accumulating in the nucleus as INI (see Fig. 5). Given the expected low frequency of frameshifts, the finding of frameshifted protein in INI as early as 12 hours after transfection argues that polyAla accumulation is a very early event in the formation of these structures. In fact, in cells transfected with as few as 14 CAGs, frameshifts are occurring and polyalanine-containing protein is accumulating in the nucleus as INI. This finding is consistent with a recent report by Perez *et al.* showing that transfection of normal sized CAG repeats leads to INI[30]. In contrast with the slow, progressive accumulation detected for the CAG/Gln constructs, transfection with the GCA/Ala constructs results in early and rapid accumulation of alanine-containing product. In this case the cells seem unable to cope with the presence of the toxic product resulting in an earlier and much more severe phenotype. The pattern of expression of polyAla products, consisting of one major juxtanuclear aggregate per cell and several smaller inclusions, is similar to that found in aggresomes, structures that form when the capacity of the proteasome degradation pathway is exceeded[31]. This seems to indicate that the direct expression of polyalanine in cells is likely to be extremely toxic. The presence of more "classical" INI in cells expressing CAG/Gln constructs is thought to result from rare frameshift events that produce alanine-containing protein, which slowly accumulates in the nucleus as aggregates. This model therefore better depicts what may be happening in diseased tissue cells.

The proposed model of toxic aggregates resulting from frameshifts into the alanine frame is consistent with previous experiments performed with MJD, as well as other exp-CAG diseases. For example, in Huntington disease (HD) and MJD, INI and protein accumulation are most clearly

detected using antibodies against epitopes to the N-terminus side of the CAG repeat[1-4], likely because frameshifting will truncate the protein shortly after the repeat. This model could also explain the correlation observed in all CAG triplet diseases between the size of the repeat and the severity of the symptoms[32]. It is suggested that if the frameshift errors occur randomly, the longer the repeat the more frequently such errors would arise. This is supported by the observations that: (1) no accumulation was yet detected in the lymphoblasts of a MJD patient with the shortest $(CAG)_{67}$ mutation; (2) fluorescence increased with the size of the CAG repeat in the transfection experiments; and (3) at any time-point, more than twice as many cells have inclusions containing frameshifted species when transfected with a $(CAG)_{82}$ construct than with a $(CAG)_{15}$ construct.

Another puzzling finding previously reported by other groups, and which can be explained by the model of frameshifts into the alternate alanine frame and the production of prematurely truncated proteins herein proposed, is the presence of short cleavage products in inclusions in HD and spinal and bulbar muscular atrophy (SBMA)[33-35]. These short products seem to be too small to be the result of caspase-3 cleavage, but are of a size consistent with premature termination of transcription or translation due to the generation of an early STOP codon in the alanine shifted frame.

The model presented herein might also explain the observation that while both CAA and CAG codons encode glutamine, only uninterrupted CAG tracts cause disease. In spinocerebellar ataxia type 1 (SCA1), for polyglutamine tracts of similar length the presence of a CAA codon interrupting a CAG tract is used to differentiate a normal from a disease causing allele[26, 36, 37]. These observations, while difficult to explain if polyglutamine is toxic, are predicted by the proposed polyAla nuclear toxicity model of the present invention, where interruption of a repeat sequence may lead to more stable transcription or translation.

Finally, the present invention may predict a similar pathogenic mechanism in the other diseases caused by expanded CAG tracts. It has been shown here that constructs containing almost exclusively a GCA tract are toxic to cells and lead to the formation of aggregates. This seems to indicate that the full MJD protein is not necessary to obtain a disease phenotype and that the presence of an expanded CAG repeat within any protein may be sufficient to cause disease. This would support the contention that the protein or gene causing the disease is indeed irrelevant to the disease process (Hardy et al. 1998). In addition, it is possible that polyAla accumulation may play a role in aging of certain cell types. For example, if frameshifts causing polyAla tracts occur with all CAG repeat-containing genes even in the "normal range" as is seen in the 14 CAG- and 37 CAG-containing constructs of the present invention, very slow accumulation of polyAla polymers may occur, leading to cell death. This could explain the observation that fewer large repeats are found in healthy elders compared to younger controls [38]. The discovery of expanded polyalanine domains in three diseases[11, 39-41] and the fact that CAG repeat frameshift mutant proteins can cause disease if they code for polyAla, pinpoints this homopolymer as a potential target for drug design. Polyalanine nuclear toxicity may well be a frequent cause of premature cell death in different tissues.

The present invention is illustrated in further detail by the following non-limiting examples.

EXAMPLE 1

Frameshifts occur in CAG tracts resulting in polyalanine-containing proteins

Two immunopurified polyclonal antisera were raised against a synthetic peptide corresponding to the 12 last amino acids predicted to result from polyAla tract producing frameshifts within the CAG repeat of the *MJD-1* gene (FS1 and FS2)(Fig. 1a). This new amino acid sequence has no homology to any known protein. Both antisera detected high molecular weight aggregates in the

stacking gels in Western blots of total lymphoblast protein from three MJD cases (Fig. 1b). In the two controls and in the MJD patient with the shortest $(CAG)_{67}$ repeat no aggregates were observed (Fig. 1b). Both FS antibodies also recognize, in all samples, an 83kD protein of unknown origin. In order to test if the signal detected in the stacking gels was the putative MJD-Ala protein, the same blots were probed with antisera raised against MJD protein epitopes (anti-ataxin-3; Fig. 1c)[17] and against polyGln domains (1C2; Fig. 1d)[18]. These results are compatible with the presence of the predicted hybrid protein in the stacking gel. An anti-ubiquitin polyclonal antibody (data not shown) also detected the accumulations, which is consistent with previous reports[1, 5, 8, 19, 20]. These accumulations are compatible with those reported by Paulson *et al.* who studied cells expressing mutant CAG tract expanded *MJD-1* using an antibody raised against the expressed ataxin-3 fusion proteins[1].

To test if the MJD-Ala protein accumulates in nuclei, immunocytochemistry on lymphoblasts from four controls and three MJD patients was performed. As Fig. 2 depicts, FS1 positive INI are observed in MJD cell lines and not in controls (Fig. 2a, b). Similar to the Western blot results, the anti-ubiquitin antibody (Fig. 2c, d) and the anti-ataxin-3 antibody (data not shown) also detect INI in MJD cell lines.

In an attempt to show the presence of the frameshifted species in affected MJD brain regions, immunohistochemical FS1 and anti-ubiquitin staining in diseased and control pons, a region known to be affected in MJD was performed (Fig. 3). Both antibodies stain intranuclear structures in neurons of this MJD brain area (Fig. 3a, c), whereas pontine neurons in control brain have no INI (Fig. 3b, d). In immunofluorescence studies, it was shown that the frameshifted product colocalizes with ubiquitin in INI of pontine neurons (Fig. 3e, f, g, h, i and j), suggesting that the intranuclear structures detected by both antibodies are the same.

EXAMPLE 2

Frameshifts into alanine frame occur preferentially with expanded CAG tracts

To test if frameshifts producing GCA/polyAla occur preferentially within expanded CAG tracts, an *in vitro* system to examine the effect of CAG repeat length on the frequency of frameshifts was designed. COS-7 cells were transfected with constructs bearing the full length *MJD-1* sequence with either a $(CAG)_{14}$, $(CAG)_{37}$ or $(CAG)_{82}$ repeat fused out of frame to the *EGFP* gene and driven by the CMV promoter (Fig. 4 and Fig. 5a). While frameshifts would occur with all CAG tracts, it was hypothesized that they would occur more frequently within larger repeats. The pMJD1, pMJD2 and pMJD3 constructs would yield EGFP-containing fusion proteins only if a frameshift occurs and produces the GCA/polyAla reading frame and the MJD-Ala protein. At 72 hours, cells transfected with pMJD2 and, especially pMJD3, showed green fluorescence (Fig. 5c, d, e). Green fluorescence was observed within 24 hours in the positive control cells transfected with the pEGFP-N1 vector alone or pMJD4 construct, where the *EGFP* coding sequence was in the glutamine frame in *MJD-1* (Fig. 5f, g). At 96 hours, and at higher magnification, frequent EGFP positive perinuclear inclusions were observed in cells transfected with the pMJD3 construct (82 CAGs) but not in the construct with 14 CAGs, and rarely in cells transfected with the 37 CAG construct (Fig. 5h). These perinuclear inclusions are similar to those found in cell culture models of MJD, as well as other CAG tract disorders, such as Huntington disease[1,4].

Western blots of protein extracted from the transfected cells were probed to confirm this interpretation (Fig. 5i, j). While 1C2 detects only the expanded MJD gene products bearing either 37 or 82 polyglutamine repeats in cells transfected with pMJD2 and pMJD3 respectively (Fig. 5i), anti-ataxin-3 detects all three different size polyglutamine-tract containing gene products (Fig. 5j), as expected. With both antibodies, protein accumulation in the wells for

pMJD3 and pMJD4 was detected. In order to further determine the nature of the accumulated protein seen for pMJD3, the pMJD1 and pMJD3 constructs were modified by adding epitopes for FS1 and HA in the alanine frame (pMJD5 and pMJD6, Fig. 4). Western blots of cells transfected with these constructs were probed with anti-HA. No signal for the 14 CAG-bearing pMJD5 construct was detected, but a band corresponding to aggregated protein was detected at the top of the gel for pMJD6, showing that with 82 repeats, frameshifts are occurring. The absence of a band corresponding to the predicted size of the ataxin-3/HA protein (Fig. 5K) indicates that all frameshifted protein is accumulating as insoluble aggregates. These experiments further demonstrate that frameshifts do occur and that their frequency increases with the size of the CAG repeat.

It shall be recognized by the skilled artisan that the *in vitro* system described above can be modified at will and still enable a determination of the frameshifting frequencies. Non-limiting examples of such modifications include the use of longer or shorter CAG-tracts, different reporter gene (i.e. luciferase) and different epitopes. In addition, such systems could be used to screen for drugs or compounds which modulate frameshifting and/or affect the level of INI formation and/or polyalanine-protein aggregation and/or cell toxicity.

It should also be recognized that the *in vivo* methods shown above (and others) could also be used to screen drugs or compounds which can modulate the level of polyalanine formation, INI formation and the like. In addition, these *in vivo* methods (and others) could be used to validate the effect of compounds or drugs identified in an *in vitro* assay.

EXAMPLE 3

Polyalanine-containing proteins are present in aggregates and are toxic to transfected COS-7 cells in a time-dependant manner

It has been shown that frameshifting into the alternate alanine frame occurs both *in vitro* and *in vivo*. To determine if these products are toxic or are simply harmless byproducts with no real consequences to the cell, a new set of *MJD-1* constructs where the reading frame immediately before the CAG repeat was mutated to code for a polyalanine stretch was designed. These constructs, pMJD9 and pMJD10, contained stretches of 14 GCA repeats and 82 GCA repeats respectively, and were fused in frame to the *EGFP* gene (Fig. 4). A HA tag was also added at the COOH-terminus in frame with the GCA stretch. COS-7 cells were transfected with these constructs, and with pMJD7 and pMJD8, which contained 14 CAG repeats and 82 CAG repeats respectively, as well as *EGFP* fused in frame with the CAG tracts. In addition, a HA epitope was also added at the COOH-terminus in frame with the GCA tracts, and so only detectable if frameshifts occurred (Fig. 4).

In a time-course experiment using anti-HA antibody as a probe to detect only protein frameshifted to the alanine frame, cells were collected and immunostained cells at 8, 12, 16, 20, 24, 48 and 72 hours. Cells transfected with the CAG/Gln constructs pMJD7 and pMJD8 showed faint background staining at 8 hours (Fig. 6a, c). Positive signal was detected for these two constructs at 12 hours (not shown) in the form of intranuclear inclusions (typically one or two per nucleus). At 20 hours, nuclei of cells transfected with pMJD7 and pMJD8 contained inclusions, but were morphologically normal (Fig. 6f, h). At 24 hours, cells transfected with the shorter construct remained morphologically normal (Fig. 6k), but cells transfected with the construct bearing 82 CAG repeats started showing some perinuclear and cytoplasmic inclusions in addition to the

intranuclear aggregates (Fig. 6m). At this time-point about 85% of transfected cells have inclusions when transfected with pMJD8, whereas only 40% of transfected cells show inclusions when transfected with pMJD7, suggesting that frameshifts occur with both constructs, but more frequently for the longer repeat. Nuclei of cells stained at 48 hours showed some membrane disintegration with both constructs, but cells containing the longer CAG repeat also had perinuclear and cytoplasmic inclusions (Fig. 6p, r), indicating a more severe phenotype. It is useful to reiterate that probing cells transfected with the CAGGln constructs with anti-HA will only detect frameshifted protein. These species are detected early in the transfection and only as INI, suggesting that, despite the probable rarity of frameshifts, they are producing proteins that accumulate as highly insoluble INI.

Inclusions for both GCA/Ala constructs (14A and 82A) as early as 8 hours after transfection were detected (Fig. 6b, d). Typically, cells have one major perinuclear or cytoplasmic inclusion and abnormal nuclear morphology. The cellular phenotype progresses rapidly with time in cells transfected with either pMJD9 (Fig. 6g, l, and q) or pMJD10 (Fig. 6i, n, s), and was extremely severe when compared with the CAG/Gln counterparts of the same repeat size (for example: compare panels m and n in Fig. 6). Cells transfected with the GCA/Ala constructs showed abnormal nuclear structure and aggregate formation mainly in the cytoplasm, usually with one major juxtanuclear inclusion and what appears to be cytoskeletal reorganization. Similar results were obtained in cells transfected in parallel with the same constructs but probed with FS1 antiserum (results not shown). At all time-points cells transfected with the pEGFP-N1 vector alone showed only background staining and were devoid of inclusions (Fig. 6e, j, o, t).

Western blots of protein extracted from the transfected cells were performed to investigate the nature of the inclusions found. Probing the blots with anti-HA detected a signal for both GCA/Ala constructs (pMJD9 and pMJD10), where the HA tag is in the main reading frame (figure 7a). No signal

was detected with equivalent exposure for the CAG/Gln constructs, but overexposure of the blots revealed the presence of smears and protein in the wells for these constructs as well. In addition, while discrete bands are resolved with pMJD8, signal is only seen in the stacking gel with pMJD10, suggesting that the larger polyAla-containing proteins are very insoluble and do not migrate into the gel at all. Probing the same blots with 1C2 confirms the presence ofpolyGln-containing proteins in the cells transfected with pMJD8, and the absence of such proteins in cells transfected with frameshifted constructs (figure 7b). The presence of signal in the stacking gel for pMJD8 may result from the presence of enough Gln residues to allow detection by the 1C2 antibody. It could also be due to the recruitment of the intact non-frameshifted protein into the insoluble aggregates, recruitment of hybrid polyAla/polyGln protein, or polyGln protein accumulation independent of polyAla polymers.

EXAMPLE 4

PolyGCA/polyAla stretches alone are toxic and sufficient for formation of aggregates

In order to determine whether a construct with only a GCA tract encoding an alanine peptide, outside the context of the ataxin-3 protein, would be sufficient to produce a cellular phenotype, a truncated $(GCA)_{42}$ construct with FS1 and HA epitopes in frame with GCA was transfected into COS-7 cells (pMJD11, Fig. 4). In this construct, the *MJD-1* sequence was truncated so that the resulting protein will only have 25 amino acid residues left upstream from the repeat, and only the FS1 and HA epitopes after the GCA tract. At 24 hours, cells showed perinuclear and cytoplasmic aggregates and an abnormal nuclear morphology (Fig. 8a), a phenotype that is very similar to the one obtained by transfection of the full-length GCA/Ala constructs described above. Mock-transfected cells showed background staining and absence of inclusions (Fig. 8b). These findings indicate that the presence of a frameshifted CAG repeat product is sufficient for toxicity, independent of the protein context, and suggest

a similar pathogenic mechanism may be operating in the other diseases caused by expanded CAG tracts. This finding is thus of relevance to expand the teachings of the present invention to all diseases or conditions caused by expanded CAG tracts and to diseases and conditions caused by the accumulation of polyalanine-containing proteins.

EXAMPLE 5

Antisera production

A 12-mer peptide corresponding to the new predicted COOH-terminus of the ataxin-3 protein after frameshift occurs (AAGPIRTEFTSM) was used to raise antisera from two rabbits, FS1 and FS2. Injections were performed using 0.5 mg of peptide conjugated to KLH and sera were collected using standard protocols[42].

EXAMPLE 6

Western Blots

For protein extraction, MJD and control lymphoblastoid cells were lysed in buffer containing NP-40. Equal amounts of protein were electrophoresed in 8% SDS-polyacrylamide gels and transblotted to nitrocellulose membranes (as commonly known). Immunodetection was performed using FS1 (1:300), FS2 (1:300), anti-ataxin-3 (1:1000), 1C2 (1:2000), anti-ubiquitin (1:400; DAKO) and FS1 and FS2 pre-immune serum (1:300). Results for FS1 and FS2 were always consistent; all experiments were repeated three times on different blots. HRP conjugated secondary antibodies were used at a 1:10,000 dilution. COS-7 cells transfected with various constructs were collected, washed and lysed in Sample Loading Buffer. 100 µg of each sample was used to run on a 10% SDS-PAGE and transblotted onto nitrocellulose membranes. Immunodetection was performed using antisera at following dilutions: 1C2 (1:5000), anti-ataxin-3 (1:2000) and anti-HA (1:1000). Results were visualized by chemiluminescence (RENAISSANCE).

EXAMPLE 7

Immunocytochemistry

MJD and control lymphoblastoid cells were harvested and a total of $50x10^4$ cells from each cell line were plated onto poly-D-lysine coated slides and fixed with acetone/methanol (1:1). Immunodetection was performed using FS1 (1:300), anti-ubiquitin (1:300), anti-ataxin-3 (1:500) and FS1 pre-immune serum (1:300). Biotinylated secondary antibodies were used at a 1:500 dilution and an amplification step was performed using the ABC kit (VECTOR). Reaction product was visualized using the VIP kit (VECTOR). Immunocyto-chemistry on COS-7 cells was performed on cover slips. At each time-point cells were fixed with 4% paraformaldehyde and immunodetection was performed using anti-HA probe (1:500) and FS1 (1:300). Secondary antibodies and subsequent amplification and detection procedures were carried out as described above.

EXAMPLE 8

Immunohistochemistry

For immunohistochemistry of brain sections, 5 µm sections of paraffin-embedded tissue from the pons of an MJD patient and a control subject were used. Sections were deparaffinized, permeabilized and immunostained with FS1 (1:50) and anti-ubiquitin (DAKO) (1:300). Biotinylated secondary antibodies were used at a 1:500 dilution and an amplification step was performed using the ABC kit (VECTOR). Reaction product was visualized using the VIP kit (VECTOR). For coimmunofluorescence of FS1 and anti-ubiquitin antibodies in brain sections, the same procedure for preparation of samples was followed. Immunodetection was performed using FS1 (1:50) and monoclonal anti-ubiquitin (ZYMED) (1:300). A mixture of Cy3-conjugated anti-mouse antibody (1:100) and fluorescein-conjugated anti-rabbit antibody (1:50) was used as secondary probe. Sections were mounted in SlowFade (Molecular Probes).

EXAMPLE 9

Plasmid construction, transfection and cell culture

DNA amplification was performed using Pfu DNA polymerase (STRATAGENE). Primer MJD-5': TTTTAAGCTTAGACAAA-TAAACATGGAG (SEQ ID NO:1) was used in conjunction with MJD-3': CCGGTGGATCCCTCATCCTGATAGGTCCCGCTGCTG (SEQ ID NO:2) for pMJD1, pMJD2 and pMJD3, or MJD-3'c: CCGGTGGATCCCTCA-TTGATAGGTCCCGCTGCTG (SEQ ID NO:3) for pMJD4 (STRATAGENE). Primer MJD-5'(HIII): TTTAAGCTTCCCACCATGGAGT-CATCTTCCA (SEQ ID NO:4) was used in conjunction with MJD-3'(BI): CCGGTGGATCCCTCAGGGCGTAGTCGGGGACGTCGTAGGGGTACATGGAT GTGAACTCTGTCCTGATAGGTCCCGCTG (SEQ ID NO:5). for pMJD5 and pMJD6, or MJD-3'c(BI): CCGGTGGATCCCAGGGCGTAGTCGGGGACGTCG-TAGGGGTACATGGATGTGAACTCTGTCCTGATAGGTCCCGCTG (SEQ ID NO:6) for pMJD7 and pMJD8, or MJD-Ala: CGGAAGAGACGAGAAGCCTACTCCGGAAAAACAGCAGCAAAA-GCAGC (SEQ ID NO:7) for pMJD9, pMJD10 pMJD11. Amplified products were digested with BamHI and HindIII and cloned into plasmid pEGFP-N1 (CLONTECH), except for pMJD11, for which the amplified product was cloned into a modified version of the pEGFP-N1 plasmid lacking the *EGFP* gene. All constructs were confirmed by sequencing (as commonly known). COS-7 cells were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum the day before transfection at 2 x 10^5 per well in 6-well plates containing sterile coverslips. COS-7 cells were transfected with plasmid DNA (2.0 µg) using lipofectamine reagent (GIBCO BRL) according to the manufacturer's instructions. For the experiment depicted in figure 4, after 72-96 hours, the cells were fixed with PBS/4% paraformaldehyde and observed under a fluorescent microscope with FITC filter in four independent experiments.

CONCLUSION

The present invention thus shows that transcriptional or translational frameshifts occurring within expanded CAG tracts result in the production and accumulation of polyalanine-containing mutant proteins. These alanine polymers might deposit in cells forming INI and lead to nuclear toxicity. Support for this disease model is provided using lymphoblast cells from MJD patients, as well as in pontine neurons of MJD brain and in *in vitro* cell culture models of the disease. Evidence that alanine polymers alone are toxic to cells is also provided and strongly suggests that a similar pathogenic mechanism underlies the other CAG repeat disorders. How these accumulations lead to cell death still needs to be elucidated.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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WHAT IS CLAIMED IS:

1. A method for the diagnosis of a disease associated with protein accumulation in intranuclear inclusions in a cell of a patient, which comprises:

a) obtaining a sample from said patient; and

b) determining a presence of said protein accumulation in said intranuclear inclusions

wherein said protein accumulation in said intranuclear inclusions is indicative of a disease associated therewith.

2. The method of claim 1, wherein said protein is a polyalanine-containing protein.

3. The method of claim 1 or 2, wherein said disease is a neurological disease.

4. The method of claim 1, 2 or 3, wherein said determining is carried out with one of a ligand and/or a nucleic acid sequence.

5. A method for the screening of agents which can modulate at least one of: (a) polyamino acid stretch-containing protein expression; (b) accumulation of polyamino acid stretch-containing proteins in intranuclear inclusions; and (c) toxicity of polyamino acid stretch-containing proteins to cells, which comprises:

a) incubating a cell which expresses a polyamino acid stretch-containing protein, associated with a disease or condition in an animal, with a compound; and

b) assessing one of a) to c);

whereby a modulator is selected when said agent significantly modulates one of said expression, accumulation and toxicity, as compared to a control agent.

6. The method of claim 5, wherein said polyamino acid stretch-containing protein is a polyalanine stretch-containing protein.

7. The method of claim 5 or 6, wherein said polyamino acid stretch-containing protein is expressed by an expression vector which comprises a repeat domain.

8. The method of claim 7, wherein said polyamino acid stretch-containing protein is a polyalanine stretch-containing protein.

9. The method of claim 8, wherein said polyalanine stretch is encoded by a CAG repeat.

10. The method of claim 9, wherein said CAG repeat is an uninterrupted CAG tract.

11. The method of claim 5, wherein said cell is selected from a lymphoblast cell from a Machado-Joseph disease (MJD) patient, a pontine neuron of MJD brain and an *in vitro* cell culture model of a neurological disease associated with said polyamino acid stretch-containing protein.

12. A method to trigger toxicity in a cell comprising an increased expression of an alanine polymer stretch in a protein.

13. The method of claim 8, wherein said polyalanine stretch is encoded by a GCG repeat.

14. The method of claim 5, wherein said cell is isolated from an oculopharyngeal muscular dystrophy (OPMD) patient.

15. The method of claim 13 or 14, wherein said GCG repeat is present in the PABP2 gene.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/18544 A1

(51) International Patent Classification[7]: G01N 33/68, C12Q 1/68

(21) International Application Number: PCT/CA00/01052

(22) International Filing Date:
8 September 2000 (08.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/152,941 9 September 1999 (09.09.1999) US

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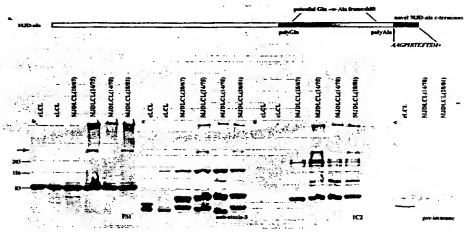
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

[Continued on next page]

(54) Title: DIAGNOSIS, PROGNOSIS AND TREATMENT OF TRINUCLEOTIDE REPEAT-ASSOCIATED DISEASES AND INTRANUCLEAR INCLUSIONS-ASSOCIATED DISEASES



(57) Abstract: The present invention relates to a method for the diagnosis of a disease associated with protein accumulation in intranuclear inclusions in a cell of a patient. The method comprises obtaining a sample from the patient; and determining a presence of the protein accumulation in the intranuclear inclusions, wherein the protein accumulation in the intranuclear inclusions is indicative of a disease associated therewith. The invention also relates to a method for the screening of agents which can module one of (a) polyamino acid stretch-containing protein expression; (b) accumulation of polyamino acid stretch-containing proteins in intranuclear inclusions; and (c) toxicity of polyamino acid stretch-containing proteins to cells, which comprises incubating a cell which expresses a polyamino acid stretch-containing protein, associated with a disease or condition in an animal, with a compound; and assessing one of (a) to (c); whereby a modulator is selected when the agent significantly modulates one of the expression, accumulation and toxicity, as compared to a control agent.

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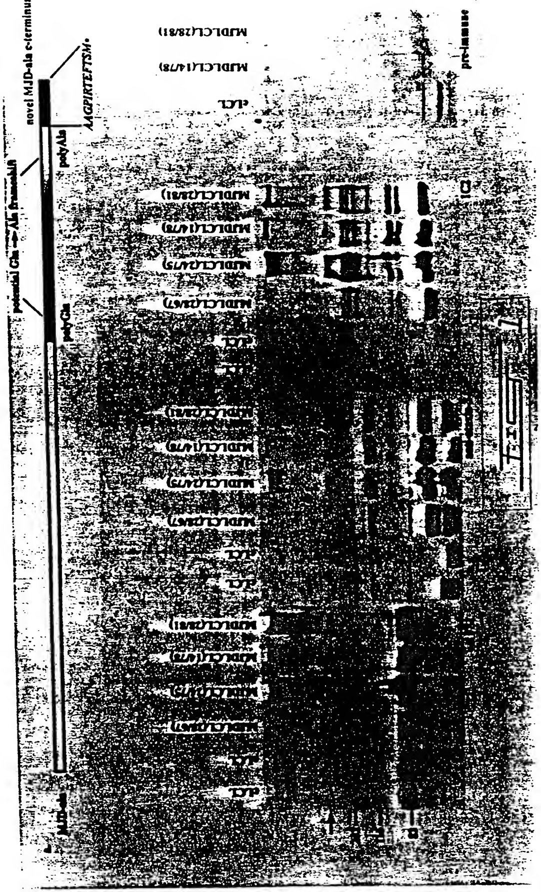
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AAGPIRTEFTSM*
polyAla
polyGln
pre-immune

FIG. 1

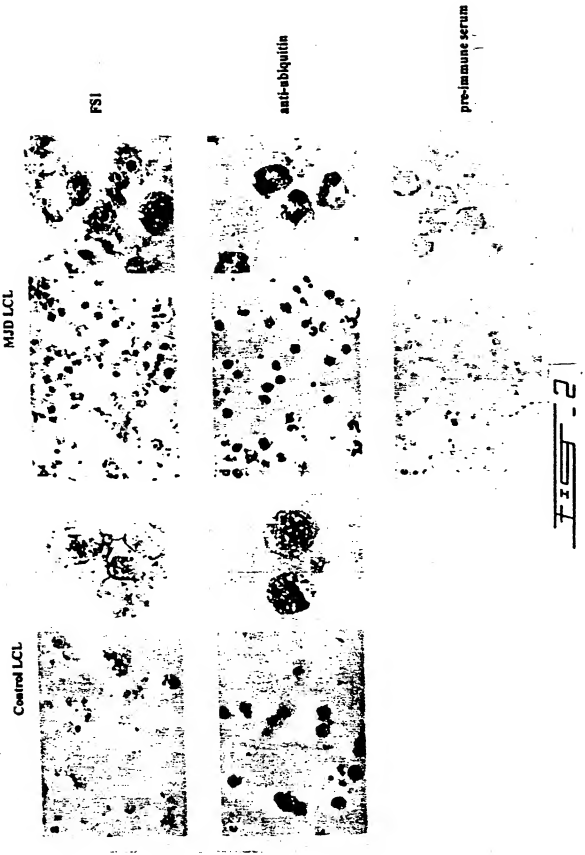


Fig - 2

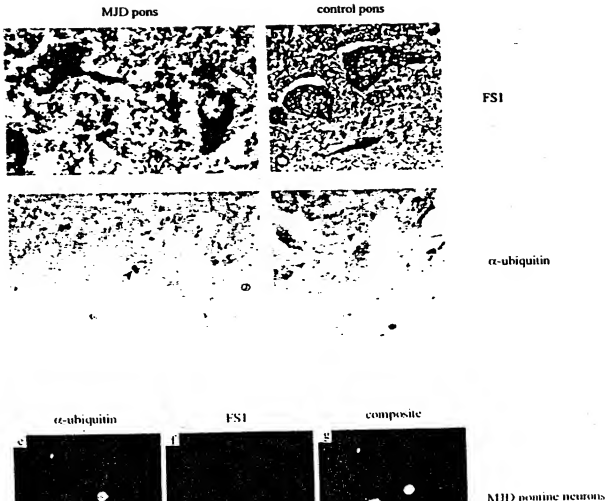
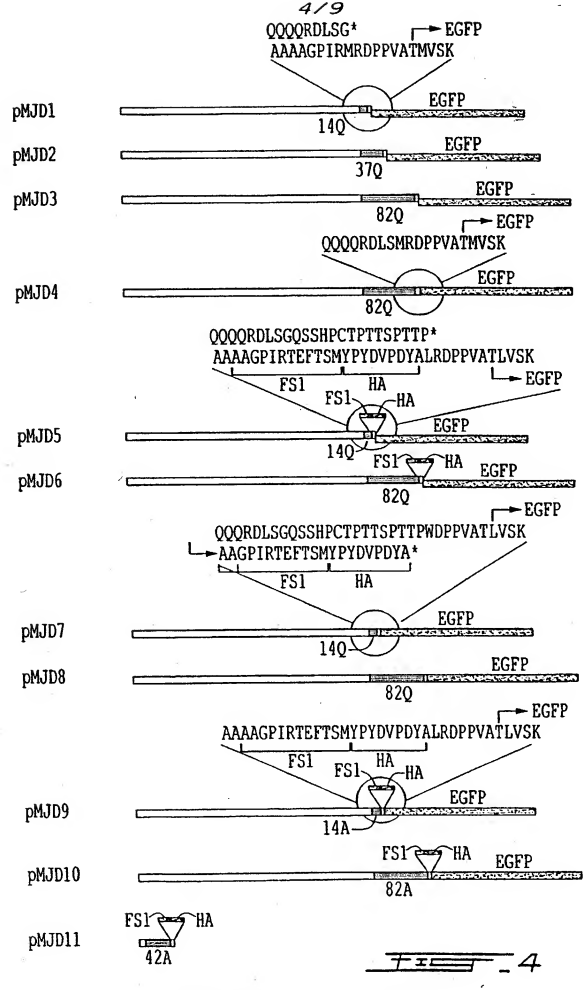

MJD pons
control pons
FS1
α-ubiquitin
α-ubiquitin
FS1
composite
e
f
g
MJD pontine neurons

FIG. 3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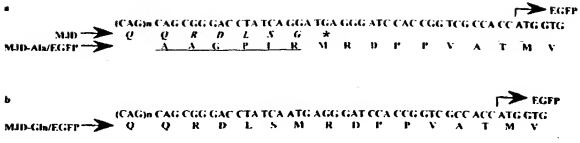
EGFP
AAAAGPIRMRDPPVATMVSK
pMJD1
EGFP
14Q
pMJD2
EGFP
37Q
pMJD3
EGFP
82Q
EGFP
QQQQRDLSMRDPPVATMVSK
pMJD4
EGFP
82Q
QQQQRDLSGQSSHPCTPTTSPTTP*
AAAAGPIRTEFTSMYPYDVPDYALRDPPVATLVSK
FS1
HA
EGFP
FS1
HA
pMJD5
EGFP
14Q
FS1
HA
pMJD6
EGFP
82Q
EGFP
QQQRDLSGQSSHPCTPTTSPTTPWDPPVATLVSK
AAGPIRTEFTSMYPYDVPDYA*
FS1
HA
EGFP
pMJD7
14Q
pMJD8
EGFP
82Q
EGFP
AAAAGPIRTEFTSMYPYDVPDYALRDPPVATLVSK
FS1
HA
FS1
HA
pMJD9
EGFP
14A
FS1
HA
EGFP
pMJD10
82A
FS1
HA
pMJD11
42A

FIG. 4


a
EGFP
(CAG)n CAG CGG GAC CTA TCA GGA TGA GGG ATC CAC CGG TCG CCA CC ATG GTG
MJD
Q Q R D L S G *
MJD-Ala/EGFP
A A G P I R M R D P P V A T M V
b
EGFP
(CAG)n CAG CGG GAC CTA TCA ATG AGG GAT CCA CCG GTC GCC ACC ATG GTG
MJD-Gln/EGFP
Q Q R D L S M R D P P V A T M V

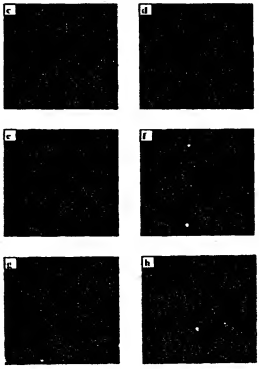
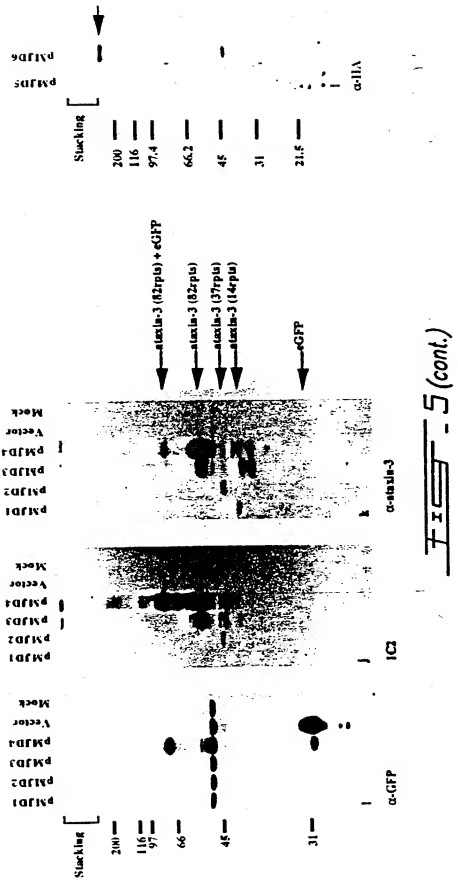

c
d
e
f
g
h

FIG 5


Stacking
200
116
97.4
66.2
45
31
21.5
pXJP6
pXJP5
α-HA
α-stanin-3
α-GFP
IC1
Mock
Vector
pXJP4
pXJP3
pXJP2
pXJP1
←stanin-3 (82rpts) + eGFP
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←stanin-3 (37rpts)
←stanin-3 (14rpts)
←eGFP

Fig. 5 (cont.)

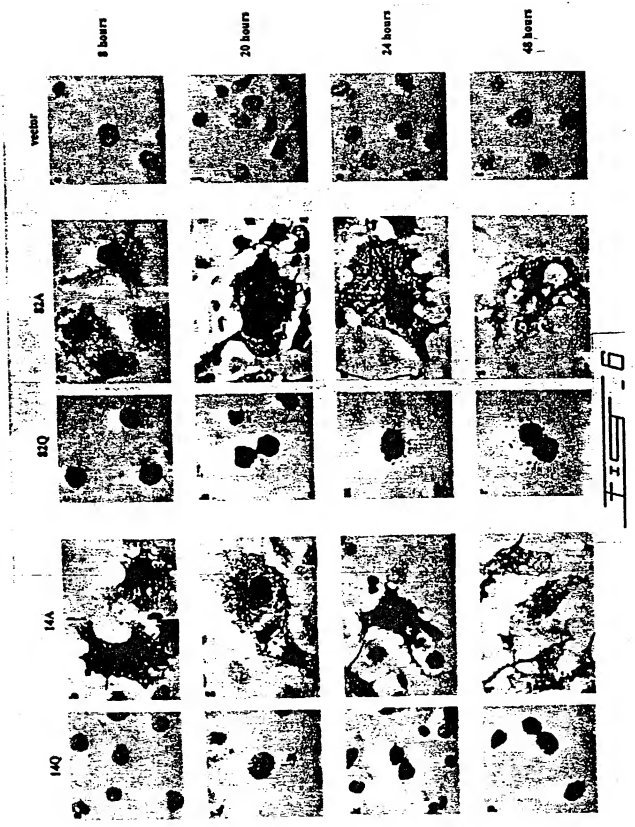

Fig. 6

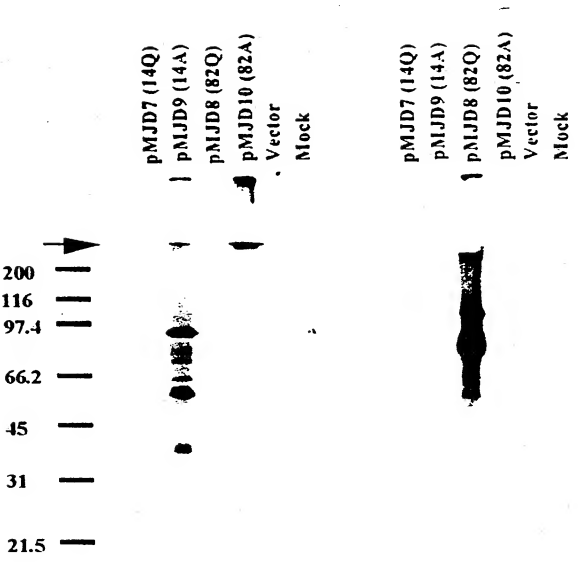

Fig. 7

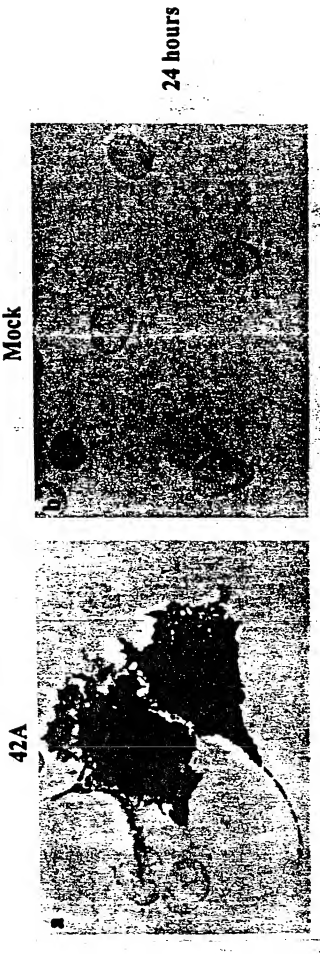

Fig. 8

Combined Declaration and Power of Attorney for Patent Application

Docket Number: 1619.0110000

As a below named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter that is claimed and for which a patent is sought on the invention entitled **Diagnosis, Prognosis and Treatment of Trinucleotide Repeat-Associated Diseases and Intranuclear Inclusions-Associated Diseases**, the specification of which is attached hereto unless the following box is checked:

☒ was filed on March 8, 2002 ;
as United States Application Number or PCT International Application Number 10/070,664 ; and
was amended on March 8, 2002 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or (f) or § 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or § 365(a) of any PCT international application, which designated at least one country other than the United States of America, listed below, and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed
PCT/CA 00/01052	PCT	September 8, 2000	☒ Yes ☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)	
			☐ Yes ☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)	

I hereby appoint the following attorney(s) and/or agent(s) associated with **CUSTOMER NUMBER 28393** to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, such attorneys currently being:

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(202) 371-2600

Appl. No. 10/070,664
Docket No. 1619.0110000

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signature of second inventor	Bernard Brais Date 02-07-29
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<120> DIAGNOSIS, PROGNOSIS AND TREATMENT OF TRINUCLEOTIDE REPEAT-ASSOCIATED DISEASES AND INTRANUCLEAR INCLUSIONS-ASSOCIATED DISEASES

<130> 1619.0110000

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1 5 10

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